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(71) Applicant *(for all designated States except US)*: **BARNES JEWISH HOSPITAL** [US/US]; 1 Barnes Jewish Hospital Plaza, St. Louis, MO 63110 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SPILBURG, Curtis, A.** [US/US]; 16052 Clarkson Woods Drive, Chesterfield, MO 63017 (US). **STENSON, William, F.** [US/US]; 11 Wakefield, St. Louis, MO 63124 (US).

(74) Agents: **DUNLAP, Charles, E.** et al.; Howell & Haferkamp, L.C., Suite 1400, 7733 Forsyth Boulevard, St. Louis, MO 63105-1817 (US).

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(54) Title: ENHANCED TRIGLYCERIDE DIGESTION IN A DEFICIENCY OF BILE SALTS

(57) Abstract: A dietary composition having enhanced digestibility of long chain triglycerides is provided where the composition includes triglycerides, the triglycerides containing fatty acid residues having at least 12 carbon atoms, and lactoferrin. The dietary composition is especially useful for infants consuming bovine milk triglycerides when human lactoferrin is used and the composition also contains human bile salt stimulated lipase.

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# ENHANCED TRIGLYCERIDE DIGESTION IN A DEFICIENCY OF BILE SALTS

## BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to methods and related compositions for enhancing the digestion of triglycerides in the presence of a deficiency of bile salts, and more particularly to methods and related compositions for enhancing the digestion of long chain triglycerides by infants and subjects having a deficiency of bile salts.

2. Description of the Related Art

For the rapidly growing infant, dietary fat in the form of triglycerides is both a major source of energy and a source of essential fatty acids that are the precursors of the eicosanoids. Birth and the first few weeks of life produce a rapid increase in the newborn's energy demands. For example, at 6 weeks of age, the infant requires 130 kcal/kg/d, with 45% of this requirement provided by fat supplied through breast milk or formula. This translates into a fat intake of approximately 6.5g/kg/d (Carey *et al.*, *Annual Rev. Physiol., 45*:651 (1983). This nutritional requirement is even more



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startling when it is realized that the energy requirements of the unborn child are largely met through carbohydrate. Thus, in a very short period after birth, the infant meets its massive metabolic needs by rapidly converting to a fat-based diet.

Human milk contains 25-30 g/L of fat, of which triglycerides are the most abundant, accounting for 99% of the total (Huisman *et al., Eur. J. of Clin. Nutr., 50*:255 - 260 (1996). While there is some variation in the fatty acid component of the triglyceride, the three most abundant are oleic acid (35%) palmitic acid (25%) and linolenic acid (15%), with stearic, palmitoleic and myristic accounting for the bulk of the others (Bitman *et al., Am. J. Clin. Nutr., 38*:300 (1983). These saturated and unsaturated 16-carbon ($C_{16}$) and 18-carbon ($C_{18}$) fatty acids, along with saturated and unsaturated 14-carbon fatty acids ($C_{14}$) and saturated and unsaturated 12-carbon fatty acids ($C_{12}$), all of which are common in milk triglycerides and in baby formula, will be referred to generally herein as "long chain fatty acids". Similarly, a triglyceride wherein each of the three fatty acid residues is a fatty acid of at least 12 carbon atoms, will be termed a "long chain triglyceride". Such long chain triglycerides are by far the most predominant fats in milk.

In spite of the fact that the ultimate goal of digestion is the absorption of fatty acid, there is very little free fatty acid in milk. Therefore, since triglycerides are not absorbed intact, the biochemical steps involved in lipid absorption are primarily designed around those enzymes and proteins that can bind to and/or hydrolyze triglycerides containing long chain fatty acids (Hernell & Blackberg, *Acta Pediatr. Suppl., 405*:69 (1994). From all these considerations, it may be seen that the digestion of the triglyceride component in milk, and especially that in human milk, plays a central role in the overall growth and development of the infant.

In the adult, the bulk of triglyceride hydrolysis occurs in the small intestine through the combined catalytic action of co-lipase dependent lipase and pancreatic cholesterol esterase. While the relative role of each of these enzymes in the overall triglyceride digestion process remains controversial, there is agreement that these two enzymes can provide complete triglyceride hydrolysis and absorption. In newborns and infants, however, this system does not pertain since neither enzyme is expressed and secreted until many weeks after birth (See, *i.e.*, Zoppi *et al., Ped. Res., 6*:880 (1972) and Lebenthal & Lee, *Pediatrics, 66*:556 (1980)). Thus, lipase activity was found to vary little from birth to the first week and it was only 2-3% of the level found

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in children older than 9 months. While its level has not been measured, it has been assumed that pancreatic cholesterol esterase also follows the same developmental pattern.

The low level of pancreatic lipolytic activity in the newborn suggests that milk itself may provide the lipases which are responsible for the complete hydrolysis of fat in the small intestine (Hernell, *Eur. J. Clin. Invest. 5*:267 (1975)). This hypothesis is especially attractive since human milk contains two lipases -- lipoprotein lipase, which is associated with milk fat, and bile salt stimulated lipase (BSSL), which remains in the soluble whey fraction (Hernell & Olivecrona, *J. of Lipid Research, 15*:637 (1974)). Because lipoprotein lipase accounts for only a fraction of the total lipolytic activity of human milk (0.5%), it is of only minor importance in the digestive process (Hernell & Olivecrona, *Id.*). On the other hand, human milk is a rich source of BSSL, 1% of total milk protein (Ellis & Hamosh, *Lipids, 27*:917 (1992)). The primary structure of this lipase was deduced from cDNA cloned from a human mammary gland library, and it was found to be identical to that for pancreatic cholesterol esterase (Hui & Kissel, *FEBS Letters, 276:*131 (1990)). Its abundance in milk and its identity to pancreatic cholesterol esterase support its role as an important component in fat digestion and absorption.

In recognition of this physiologic function, it has been proposed to fortify milk, especially cow's milk, with BSSL as a means to augment the digestibility of milk fat (Tang & Wang, in U.S. Patent No. 4,944,944). Moreover, using the nucleotide sequences from BSSL, transgenic animals have been described that express human BSSL in their milk (U.S. Patent Nos. 5,763,739 and 5,716,817).

BSSL requires an essential activator, micellar concentrations of bile salt, to maintain maximum hydrolytic activity toward long chain triglycerides (Wang *et al., J. Biol. Chem. 256*:10198 (1983)). The two main components of bile salts are sodium glycocholate and sodium taurocholate. It has been shown that when the sodium taurocholate concentration is below 4 mM, the enzyme does not hydrolyze triolein (Hernell & Blackberg, *Ped. Res., 16*:882 (1982)). If it were not for the absolute requirement for bile salt for the hydrolysis of long chain triglycerides, the presence of high concentrations of BSSL in breast milk would lead to premature hydrolysis of triglycerides in the lactating breast cell or in the infant's stomach. While this requirement insures that triglycerides are hydrolyzed at the appropriate time and in the

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appropriate place, the necessity of micellar concentrations of bile salt places another biochemical demand on the infant's digestive system that must be met in order for efficient fat absorption to occur.

In view of the central role that bile salt plays in adult and infant digestion, it is surprising that its concentration is so different in the early stages of life when compared to that of the adult. For example, the adult intraluminal bile salt concentration ranges from 7.0 mM to 20.0 mM, but in infants the comparable value is 1.5 to 5.0 mM (Watkins, *Pediatrics, 75 (suppl)*:151 (1985); Norman *et al., Acta Ped. Scand., 61*:571 (1972); Heubi *et al., J. Lab Clin. Med., 100*:127 (1982)). Moreover, when appropriate correction is made for body surface area, the rate of synthesis of bile salts in infants is less than that for adults (Watkins *et al., N. Engl. J. Med., 288:*431 (1972)). Taken together, these results indicate that, at least in the first few months of life, the infant provides only a limited amount of bile salt, a critical component that is required for the efficient hydrolysis and subsequent absorption of dietary fat. This occurs at precisely the time when the newborn is confronted with a high fat diet and when high concentrations of milk-derived BSSL are present.

Accordingly, it would be desirable to provide a way to enhance the digestibility of triglycerides, especially long chain triglycerides, by infants and other subjects who depend upon the hydrolytic activity of BSSL, but who have low levels of bile salts. It would also be desirable to provide a way to use long chain triglycerides from an inexpensive and readily available source - cow's milk, for example - as a highly digestible source of dietary fats for infants and such subjects. It would also be desirable to provide dietary compositions that provide enhanced digestibility of long chain triglycerides for such infants and subjects.

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BRIEF SUMMARY OF THE INVENTION

Briefly, therefore, the present invention is directed to a novel dietary composition having enhanced digestibility of long chain triglycerides by infants and other subjects with a deficiency of bile salt. The composition comprises long chain triglycerides and lactoferrin.

The present invention is also directed to a novel human infant formula having enhanced fat digestibility comprising bovine milk triglycerides and isolated human lactoferrin.

The present invention is also directed to a novel human infant formula having enhanced fat digestibility comprising soy milk triglycerides and isolated human lactoferrin.

The present invention is also directed to a novel fortified milk composition comprising long chain triglycerides from a first source and isolated lactoferrin from a second source in an amount sufficient to result in a rate of hydrolysis of the triglycerides when the fortified milk composition is contacted with BSSL and bile salt that is double what results if a lactoferrin-free composition that is otherwise identical to the fortified milk composition is contacted with BSSL and bile salt.

The present invention is also directed to a novel method for enhancing the digestion and absorption of long chain triglycerides, the method comprising fortifying the triglycerides with an amount of lactoferrin sufficient to double the rate of hydrolysis of the triglycerides over the rate obtained in the absence of lactoferrin, when such triglycerides and lactoferrin are in the presence of bile salt stimulated lipase and in the presence of bile salt.

The present invention is also directed to a novel method for treating a subject having a bile salt deficiency comprising administering to the subject, in conjunction with the oral ingestion of long chain triglycerides, bile salt stimulated lipase and isolated lactoferrin in an amount sufficient to double the rate of hydrolysis of the triglycerides over the rate obtained in the absence of lactoferrin.

The present invention is also directed to a novel method for feeding an infant a dietary base from a first source, which dietary base contains long chain triglycerides, the method comprising administering bile salt stimulated lipase and human lactoferrin to the infant in amounts sufficient to improve the infant's digestion and absorption of the triglycerides, wherein the lactoferrin is from a second source.

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The present invention is also directed to a novel method for preparing a dietary composition having enhanced digestibility of long chain triglycerides comprising mixing with such triglycerides an amount of lactoferrin sufficient to improve the digestion and absorption of the triglycerides.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of a way to enhance the digestibility of triglycerides, especially long chain triglycerides, by infants and other subjects that depend upon the hydrolytic activity of BSSL, but have low levels of bile salts; the provision of a way to use long chain triglycerides from an inexpensive and readily available source - cow's milk or soy milk, for example - as a highly digestible source of dietary fats for infants and such subjects; and the provision of dietary compositions that provide enhanced digestibility of long chain triglycerides for such infants and subjects.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a plot of the protein content - indicated by absorbance at 280 nm - and the hydrolytic activity against p-nitrophenylbutyrate of fractions obtained from separation of human breast milk by chromatography on S-Sepharose;

Figure 2 shows the sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of lactoferrin (in the left lane) compared with molecular weight standards (in the right lane) of 112 kDa, 84 kDa, 53 kDa, 29kDa and 14.4kDa; and

Figure 3 is a plot of the triolein hydrolysis activity of bile salt stimulated lipase versus the concentration of taurocholoate at various levels of lactoferrin.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, it has been discovered that the presence of lactoferrin permits bile salt stimulated lipase to hydrolyze long chain triglycerides efficiently in the presence of a deficiency of bile salt. This surprising and previously unrecognized property of lactoferrin provides a method by which dietary compositions that contain long chain triglycerides, even triglycerides derived from bovine milk, can become highly digestible sources of dietary fats for infants and other subjects deficient in bile salts by fortifying the composition containing the triglycerides with lactoferrin. It is important only that the lactoferrin that is used is

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one that enhances the rate of long chain triglyceride hydrolysis by the BSSL with which it comes in contact. It has been found, for example, that human lactoferrin enhances the activity of human bile salt stiumlated lipase, but it does not enhance the activity of porcine cholesterol esterase.

It has been found to be useful in some cases to supplement the dietary composition additionally with BSSL, so that the ingested triglycerides are accompanied by supplemental BSSL as well as supplemental lactoferrin.

Without wishing to be bound by this or any other theory, it is believed that the presence of lactoferrin enhances the ability of BSSL to hydrolyze triglycerides, especially long chain triglycerides, efficiently in the presence of sub-micellar concentrations of bile salt. BSSL plays an essential role in the hydrolysis and subsequent absorption of fat, but bile salt in a micellar concentration is thought to be an essential activator and necessary to maintain maximum BSSL activity. The infant cannot meet this requirement because its bile salt concentration is below the critical micelle concentration; however, human milk contains lactoferrin that is believed to enable BSSL to hydrolyze long-chain triglycerides at lower bile salt concentrations. Therefore, addition of human lactoferrin at a concentration similar to that found in human milk, either alone or in combination with BSSL, can enhance fat absorption.

It is also believed that the administration of human lactoferrin, either alone or in combination with BSSL, to those individuals suffering from nutrient malabsorption due to pancreatic insufficiency and/or bile salt deficiency can enhance their hydrolysis of triglycerides and absorption of dietary fats.

Lactoferrin:

Lactoferrin is a glycoprotein that has an average molecular weight of about 77 kilo Daltons (kDa) and can reversibly bind iron. Lactoferrin is a major protein component of milk, especially human breast milk, and is present at about 1 mg/mL to about 7 mg/mL in human milk. Although it has been suggested that the protein plays either a role in iron metabolism or in the regulation of microflora in the intestine (Iyer & Lonnerdal, *Eur. J. of Clin Nutr., 47*:232 (1993); Sanchez *et al., Arch. of Dis. in Childhood, 67*:657 (1992)), these functions have not been supported by in vivo studies. In a brief report, lactoferrin was shown to bind to BSSL and to produce modest enhancement of the hydrolysis of p-nitrophenylacetate, a non-pysiological

substrate, and cholesteryl oleate, a substrate that is present in only small quantities in milk (Erlanson-Albertsson, *et al., Biochim. Biophys. Acta, 829*:282-287 (1985)). This work also examined the effect of lactoferrin on the BSSL-catalyzed hydrolysis of tributyrin, a short-chain triglyceride that does not require taurocholate (non-essential activator) for hydrolysis (Wang *et al., J. Biol. Chem., 258*:9197 (1983)). However, this work did not include the hydrolysis of long-chain triglycerides, such as those commonly found in milk and it did not explore the significance of the interaction between BSSL and lactoferrin on the lipase's critical capacity to hydrolyze triglycerides of any sort in the presence of varying concentrations of bile salt.

Lactoferrin that is useful in the present invention can be derived from any source. The preferred lactoferrin, however, is of a type that is sufficient to enhance the hydrolytic activity of BSSL on long chain triglycerides. That is to say that the preferred lactoferrin has the capability of enhancing the hydrolysis of a long chain triglyceride by BSSL into fatty acids and a diglyceride, monoglyceride, or glycerol. It is more preferred that the lactoferrin be derived from the same source as the BSSL. It is most preferred that both the lactoferrin and the BSSL are derived from the same species as the subject that is to consume the dietary compositions or is to be treated by the methods of the invention.

When it is said that the lactoferrin is "derived from" a source, what is meant is that the lactoferrin is isolated or purified from that source, or that genetic material from that source has been expressed in a recombinant organism to produce the lactoferrin. Lactoferrin that is "derived" from a source is chemically the same, or substantially the same, as the lactoferrin that is present in or produced by the source itself. Thus, the lactoferrin that is purified or isolated from human breast milk, for example, which could be referred to herein as lactoferrin derived from human milk, or human lactoferrin, would be substantially the same in chemical structure as the lactoferrin present in fresh, unmodified human breast milk. Likewise, lactoferrin produced from the expression in a recombinant microorganism or transgenic animal of a gene that codes for the production of human lactoferrin would have the same chemical structure as, and could also be referred to as lactoferrin derived from human milk, or human lactoferrin (hLF).

Methods for the isolation of lactoferrin from milk are well known and have been disclosed in U.S. Patent Nos. 5,596,082; 5,516,675; 5,149,647; 4,997,914;

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4,791,193; and 4,668,771. The technical literature also provides numerous methods for the isolation of lactoferrin from milk. Several of these methods have been described by Querinjean *et al.* in *Eur. J. Biochem., 20*:420 (1971); by Johannson in *Acta Chem. Scand. 23*:683 (1969); by Johannson *et al.* in *Nature 181*:996 1958); by Torres *et al. in Biochem. Biophys. Acta 576*:385 (1979); and by Ekstrand and Bjorck, in *J. of Chromatography 358*:429 (1986).

Alternatively, lactoferrin can be produced by expressing exogenous genes in recombinant microorganisms as described in U.S. Patent Nos. 5,849,881; 5,766,939; 5,571,896; 5,571,697; and 5,571,691, or in transgenic animals as described in U.S. Patent Nos. 5,861,491 and 5,849,885. Additional methods for processing and/or purifying lactoferrin are disclosed in U.S. Patent Nos. 5,340,924 (heat treatment for sterilization); 5,317,084 (chromatographic separation from impurities); 5,141,743; and 5,116,953 (thermal treatment).

One method that can be used for the production of substantially pure human lactoferrin from milk, especially from the milk of transgenic animals, is described by Nuyens *et al.* in U.S. Patent No. 5,849,885. Briefly stated, the method discloses that the milk containing endogenous and human lactoferrin is contacted for a suitable binding period with a strong cation exchange resin under elevated ionic strength conditions which are at least 10 mM higher in monovalent cation concentration than milk. After the human lactoferrin is allowed to form complexes with the resin, the unbound fractions of the milk are removed from the resin. The human lactoferrin is then eluted from the resin with a salt solution having an ionic strength sufficient to elute the human lactoferrin and a eluant solution is obtained that contains the human lactoferrin and is substantially free of the endogenous lactoferrin.

Although lactoferrin can be used in the present invention in almost any form, it is preferred that the lactoferrin be concentrated from the levels at which it normally occurs in milk. It is more preferred that the lactoferrin be isolated from other components with which it is normally found. For example, it is preferred that the lactoferrin be concentrated to the point where the lactoferrin is at least about 25% pure lactoferrin, by weight of dry substance; or more preferred that it be at least about 50% by weight lactoferrin; even more preferred that it be at least about 80% lactoferrin; yet more preferred that it be at least about 95% lactoferrin; and most preferred that it be substantially pure lactoferrin. By "substantially pure", it is meant

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that the lactoferrin comprises at least about 98% lactoferrin by weight of dry substance.

The lactoferrin that is useful in the invention is not required to be pure and can be accompanied by other compounds. It is preferred, however, that the lactoferrin is not contaminated by any compound that is an inhibitor for BSSL, or that is toxic or otherwise harmful to the infant or subject that is to ingest the material. It is also preferred that the lactoferrin that is used in the invention be in physiologically active form and not to have been denatured by thermal, chemical or physical treatment.

Bile Salt Stimulated Lipase

An important feature of this invention discovered by the subject inventors is that lactoferrin has been found to enhance the activity of BSSL under certain conditions. As discussed previously, BSSL is a normal component of the milk of only a few species; namely, humans, gorillas, cats and dogs (Freed *et al., Biochim. Biophys. Acta, 878*:209-215 (1986)). However, it is not present in the milk of cows, goats, rats, rabbits, horses, or pigs (Blackberg *et al., FEBS Lett.* 112:51-54 (1980); Freudenberg, *Experimentia 22*:317 (1966)).

As used herein, the terms "bile salt stimulated lipase" or "BSSL" will be understood to mean the enzyme that is present in milk, while the terms "cholesterol esterase", or "pancreatic carboxylesterase" can be used interchangeably and refer to the lipase that is produced by the pancreas. Because BSSL and cholesterol esterase have the same, or substantially the same primary structure, and differ only in the type of glycosylation, they are believed to respond similarly to the enhancing action of lactoferrin. Therefore, it is believed that the present invention can be successfully operated with either of these two enzymes and, accordingly, when one of these is mentioned, it is to be understood that it is believed that the other could also be used in that situation.

BSSL can be purified from natural sources, such as the milk of human and certain animal species, or can be isolated from the small intestine or from pancreatic juice. A method of isolating BSSL from milk is described by Wang *et al.*, in *Anal. Biochem., 133*:457-461 (1983). However, it may be less expensive to produce the enzyme by genetic engineering using standard recombinant DNA techniques for the translation and expression of the BSSL gene in microorganisms or in transgenic

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animals. Such genetic engineering methods have been described in U.S. Patent Nos. 5,827,683; 5,763,739; and 5,716,817. As mentioned before, it is preferred that the BSSL be derived from the same source as the lactoferrin that is used in the invention and most preferred that both the lactoferrin and the BSSL be derived from the same species of subject to which the compositions of the invention are to be administered or fed. In other words, if a dietary composition containing an embodiment of the invention is to be fed to human infants, it is most preferred that the lactoferrin and the BSSL be derived from a human source.

In those embodiments where BSSL is a component of the novel dietary composition, or is used in the novel methods of this invention, it can be used in a crude or a purified form, as long as it is not accompanied by contaminants that are inhibitors to its activity, or are toxic or otherwise harmful to the infant or subject to which it is administered. It is preferred that BSSL be used in a purified or isolated form. As used herein, "isolated BSSL" is to be understood to mean BSSL that is at least about 50%, by weight, pure BSSL. It is preferred that isolated BSSL be at least about 80% by weight, pure BSSL; more preferred that it be at least about 95% by weight, pure BSSL; and most preferred that it be substantially pure BSSL. By "substantially pure" it is meant that the BSSL be at least about 98% by weight, pure BSSL.

As mentioned before, it is known that BSSL is activated or accelerated in the presence of bile salts. But it has surprisingly been found that when the BSSL enzyme is hydrolyzing long chain triglycerides, this activation is not a linear function of bile salt concentration, but that the activation effect is enhanced markedly when the bile salt concentration is approximately equal to the critical micelle concentration of the bile salt.

The bile salt concentration in the normal adult human small intestine is believed to be about 6 mM to about 10 mM. However, the bile salt concentration in newborn human infants is about 1 mM to about 3 mM. Under the conditions in the small intestine, it is believed that the critical micelle concentration (CMC) for bile salt is about 3 mM to about 4 mM. As used herein, the "critical micelle concentration" or "CMC" for bile salt is to be understood to be the minimum concentration of bile salt in the small intestine of a subject at which micelles are formed that contain the fats that are present in the small intestine. The inventors have found that the hydrolysis of

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long chain triglyderides into component glycerol and fatty acids by BSSL in the presence of bile salts proceeds very slowly, or not at all, at bile salt concentrations that are below the CMC, or below about 3 mM to about 4 mM. As used herein, a "deficiency" of bile salt is to be understood to be a condition where bile salt is present at levels below the CMC in the small intestine. However, as the bile salt concentration is increased to the CMC and beyond, the rate of hydrolysis accelerates substantially until it reaches a maximum rate at a bile concentration of around 5 mM to about 7 mM. A further increase in the bile salt concentration beyond that level is not thought to result in a substantial increase in hydrolysis rate. This is important to the present invention because it indicates that the bile salt level in the small intestine of the human infant is below that preferred for the digestion of milk triglycerides.

It has surprisingly been found that lactoferrin apparently acts synergistically with bile salt to greatly accelerate the hydrolysis rate of triglycerides, especially long chain triglycerides, by BSSL at bile salt levels that are below the CMC. Thus, human infants that receive both BSSL and lactoferrin in breast milk are able to digest milk triglycerides. However, human infants who have not developed the capacity to produce sufficient endogenous lipases and who are deficient in bile salt, who do not receive both BSSL and lactoferrin in their diet, are not able to digest these triglycerides. One great advantage of the invention, not obtainable heretofore, is to be able to ensure enhanced digestion by infants of long chain triglycerides from any source, but particularly from a readily available and inexpensive source -- cow's milk, for example -- by supplementing the milk with lactoferrin and BSSL from a second source or sources -- that is, a source or sources other than the source of the triglycerides; for example, human lactoferrin and human BSSL may be used.

Dietary Composition

The present invention can be used to provide a dietary composition having enhanced digestibility of long chain triglycerides. The composition includes long chain triglycerides and lactoferrin. It is preferred that the long chain triglycerides be from a first source and that the lactoferrin be from a second source. When the term "composition" is used herein, what is meant is a mixture formed by combining two or more ingredients. Human breast milk without supplementation or modification, for example, is excluded from being a composition, as the term is used herein, because

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there is no act of combining ingredients. When it is said that components of the composition are supplied by different sources -- a first and a second source -- this should not be taken to mean that the named components -- lactoferrin and triglycerides, for example -- must all be supplied by separate sources. Such a dietary composition can include lactoferrin and triglycerides from one source, or sources, and triglycerides from a different source, or sources. By way of example, the dietary composition may be milk containing long chain triglycerides with added lactoferrin.

If the composition is to be fed to infants who are also deficient in lipases, the composition preferably also contains BSSL, preferably from a source or sources other than that of the triglycerides. If the composition is to be fed to human infants, it is preferred that the lactoferrin be human lactoferrin and that the BSSL be derived from a human source as well. It is more preferred that the lactoferrin be isolated lactoferrin; and even more preferred that it be isolated human lactoferrin. It is also preferred that the lactoferrin be produced by a recombinant organism. The organism can be either a microorganism or a transgenic animal. It is more preferred that human lactoferrin and the human BSSL be produced in the milk of a transgenic animal.

It is understood that adding any amount of lactoferrin to a diet containing long chain triglycerides can enhance the digestibility of such triglycerides by an infant or by a subject that is deficient in bile salt. However, it is preferred that lactoferrin be added to a dietary composition containing long chain triglycerides in an amount sufficient to enhance the rate of hydrolysis of the triglycerides when the composition is ingested by a human infant or other subject that is deficient in bile salt; and it is more preferred that lactoferrin be added in an amount sufficient to at least double the rate of hydrolysis of the triglycerides; and even more preferred that the amount be sufficient to at least double the hydrolysis rate of the triglycerides when they are ingested by a human infant. It is most preferred that the lactoferrin be present in the composition in an amount that provides a lactoferrin concentration of at least about 1 mg/ml in the small intestine of a human infant upon its ingestion of a normal amount of the dietary composition.

By "subject", it is meant a mammal that produces the bile salts of sodium taurocholate and/or sodium glycocholate and has such salts present in its small intestine. By "optimum hydrolysis" it is meant the maximum hydrolysis rate of triglycerides for the amount of BSSL that is present.

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When it is said that the hydrolysis rate of triglycerides is "doubled", it is meant that the rate of hydrolysis of triglycerides by BSSL in the presence of the added lactoferrin is at least twice the rate of hydrolysis of the same triglycerides by BSSL under the same conditions except free of the added lactoferrin.

It is preferred that the long chain triglycerides of the composition be derived from milk; and more preferred that they be derived from bovine milk. As an alternative, however, the triglycerides can be derived from non-milk sources and, in fact, from plant sources for purposes of reducing the cost of the composition.

When BSSL is used in the composition, it is preferred that the BSSL be isolated BSSL; and even more preferred that it be isolated human BSSL. The BSSL that is produced by a recombinant organism can also be used in the composition. As described above for the lactoferrin, the organism can be either a microorganism or a transgenic animal. Almost any amount of BSSL can be used in the composition with positive results, but it is preferred that an amount of BSSL be used that provides approximately the same amount of BSSL as provided by the normal breast milk diet of a breast-fed human infant.

The composition can be used in almost any physical form. It can be a solid, a paste, a gel, or a liquid. It is only necessary that it be in a form that is suitable for ingestion by the infant or the subject to which it is to be fed or administered.

One embodiment of the invention is a human infant formula having enhanced fat digestibility, where the formula includes bovine milk triglycerides and isolated human lactoferrin. The formula can further include isolated human BSSL. The lactoferrin and BSSL can be included in the formula in the amounts described above for the dietary composition.

Another embodiment of the invention is a fortified milk composition that includes long chain triglycerides, which may be either milk or plant, and isolated lactoferrin in an amount sufficient to double the rate of hydrolysis of the triglycerides when the fortified milk composition is contacted with BSSL and bile salt. It is preferred that the triglycerides are from a first source and that the isolated lactoferrin is from a second source. If the milk to be fortified is free of BSSL, isolated BSSL can also be added as a supplement.

Although only the novel components of the dietary composition, the human infant formula and the fortified milk composition are described above, it is to be

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understood that such compositions can also contain many other materials. For example, additional proteins, carbohydrates and lipids can be included along with various additives, such as anti-oxidants, emulsifiers, stabilizers, vitamins, color enhancers, flavors, flavor enhancers, pigments, preservatives, and any other material that is approved for use in foods or formulas of the same type as those of the invention.

Preparation of the compositions

The dietary composition that has enhanced digestibility of long chain triglycerides can be prepared by mixing together the long chain triglycerides and the lactoferrin. If BSSL is to be included in the composition, it can be intermixed with the first two ingredients at the same time or at a later time. The mixing can be carried out in any equipment that is normally used for the mixing of solid or liquid food ingredients, as the case may be, and the composition can be used directly after mixing, or can be packaged for storage, transfer and later consumption. Recognized food-grade equipment and procedures should be used throughout the processing and packaging of the composition to ensure that a product reaches the final consumer in a form that is safe for consumption.

Methods for using the invention

The present invention can be used in several advantageous ways to enhance the digestion of long chain triglycerides by infants or subjects having a deficiency of bile salts. One embodiment provides an advantageous method for feeding an infant a dietary base having long chain triglycerides from a source that can be other than human milk. The method includes feeding an infant a dietary base from a first source, which base contains long chain triglycerides, the method comprising administering BSSL and human lactoferrin to the infant in an amount sufficient to improve the infant's digestion and absorption of free fatty acids derived from hydrolyzed triglycerides, wherein the lactoferrin is from a source different from that of the triglycerides. The triglycerides may be from bovine milk, for example, or can be from soy protein or other plant sources, or a blend of both. It is preferred, however, that the BSSL and the lactoferrin be from a human source.

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The triglycerides, BSSL and lactoferrin can be mixed together with any other desirable infant formula ingredients and packaged, prepared for consumption and consumed the same as with any conventional infant formula. Upon contact with bile salt in the small intestine of the infant, the supplemental BSSL and lactoferrin result in significantly increased hydrolysis of the triglycerides and increased absorption of the fat. This increased fat absorption provides substantially increased nutritional benefits for the infant.

Another embodiment is a method for enhancing the triglyceride hydrolytic activity of BSSL in the presence of a deficiency of bile salt. This is especially useful for treating subjects having a bile salt deficiency. In this case the invention, as described above, can be applied to non-infant subjects who have a bile salt deficiency. Such a deficiency can be caused by a disease condition, or may be a permanent or temporary consequence of surgery. For example, in those situations where gall stone or gall bladder removal causes a cessation or diversion of bile salt introduction into the small intestine, the subject may become deficient in bile salts. The addition of lactoferrin and optionally BSSL to these subjects can enhance their ability to digest dietary triglycerides.

The utility of the invention is not limited to infants, or, indeed, to humans, but can be useful for subjects of any species in which digestion of triglycerides is important for nutritional purposes as long as the species produces bile salts in its digestive tract. In these embodiments, lactoferrin and optionally BSSL can be administered to the subject as a portion of the diet or by any other means of administration of the ingredients to the location in the digestive tract where bile salts occur and where triglyceride hydrolysis normally occurs. Such administration produces the advantageous enhancement of triglyceride hydrolysis and fat absorption as described above.

The following examples describe preferred embodiments of the invention. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

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## EXAMPLE 1

This example demonstrates that human milk contains a substance that activates the BSSL catalyzed hydrolysis of p-nitrophenyl butyrate (pNPB) (Hosie *et al., J. Biol. Chem., 262:*260 (1987)). Human milk (75 mL) was prepared for chromatography by adjusting its pH to 5.1 by the drop-wise addition of acetic acid. The solution was then centrifuged and filtered through cheesecloth and then through a 0.8 μm filter. A purification procedure developed for pancreatic cholesterol esterase was followed (Spilburg *et al., Biochemistry 34*:15532 (1995)). After applying the acidified whey to S-Sepharose equilibrated with 25 mM acetate pH 5.1, the resin was washed with 300 mM NaCl, 25 mM acetate pH 5.1. The enzyme was then removed with a salt gradient increasing from 300 mM NaCl, 25 mM acetate pH 5.1 to 1.0 M NaCl, 25 mM acetate pH 5.1. As shown in Figure 1, enzyme activity eluted as a well-defined peak at about 500 mM NaCl, 25 mM acetate pH 5.1; however, only $3.99 \times 10^5$ activity units were recovered of $2.29 \times 10^6$ activity units applied (17% recovery). To verify that this activity loss was due to the chromatographic removal of an activator, aliquots from the column wash-through and from the salt gradient were incubated with purified BSSL to probe for increased esterolytic activity. None of the pre-gradient column washes had any effect on BSSL hydrolytic activity. On the other hand, when aliquots from fractions of the large protein peak that eluted after BSSL were incubated with purified enzyme, there was a marked enhancement of activity. As shown in Table 1 below, fraction 84 increased the activity almost 3-fold.

Table 1: Effect Of Column Fractions On BSSL Catalyzed Hydrolysis Of p-Nitrophenylbutyrate

| Condition* | Activity, nmole pNPB hydrolyzed/min/5μL Fraction** | |
|---|---|---|
| | Fraction Without BSSL | Fraction With BSSL |
| BSSL added | -- | 9.1 |
| BSSL + Fraction 82 | 0.0 | 19.8 |
| BSSL + Fraction 84 | 0.0 | 25.5 |
| BSSL + Fraction 86 | 0.0 | 16.4 |

*See Figure 1 for fraction numbers

** The change in absorbance was followed at 400 nm and converted to nmoles of p-nitrophenol using the extinction coefficeint, $\varepsilon = 14775 \times 10^4$ L mole$^{-1}$ cm$^{-1}$ (Jackson *et al., FEBS Lett., 190*:297 (1985).

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## EXAMPLE 2

This example identifies the activator of the BSSL catalyzed hydrolysis of p-nitrophenyl butyrate hydrolysis as lactoferrin. Fractions that activated BSSL were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and a single band was found at molecular weight 83 kDa (Figure 2). Since the protein was pure, the first nine N-terminal amino acids were determined and found to be $NH_2$-G-R-R-R-R-S-V-Q-W, which are identical to those of the N-terminus of human milk lactoferrin. Lactoferrin is an iron-containing protein that has a metal-related absorbance at 465 nm and, moreover, the protein isolated here also possessed this unique spectral feature. By determining the ratio of the absorbance at 465 nm to that at 280 nm, a standard procedure for assessing metal content (Mazurier & Spik, *Biochem. Biophys. Acta, 629*:399 (1980)), the protein was found to contain 12±3% Fe, a value that is frequently found for lactoferrin isolated from milk. To verify further that the protein isolated here was lactoferrin, a Western blot using antibody to the human protein showed only a single band at 83 kDa (data not shown). Finally, commercial human lactoferrin was added to purified BSSL and a similar enhancement of activity was found. Thus, by molecular weight, N-terminal analysis, cross-reactivity with antibody to lactoferrin, iron content and kinetic correspondence to commercially available material, the activator of BSSL hydrolysis was identified as lactoferrin.

## EXAMPLE 3

This example demonstrates that human lactoferrin interacts specifically with human BSSL. To determine the species specificity of this interaction, pig pancreatic cholesterol esterase was first purified as described elsewhere (Spilburg *et al., Biochemistry, 34*:155321 (1995)). Human lactoferrin was then incubated either with purified pig pancreatic cholesterol esterase or with human BSSL, and the hydrolytic activity was measured by addition of an aliquot to 1.0 mL of 0.5 mM p-nitrophenylbutyrate. For porcine cholesterol esterase, increasing lactoferrin from 0 to 32 μg in the assay mixture decreased its catalytic activity from 81.0 μmole/min/mg enzyme to 63.4 μmole/min/mg enzyme. On the other hand, when lactoferrin was

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increased from 0 to 20 μg in the BSSL assay, the activity increased about 3-fold from 6.2 μmole/min/mg enzyme to 18.2 μmole/min/mg enzyme. At 50 μg lactoferrin, the activity decreased to 13.1 μmole/min/mg enzyme, a value that was still 2-fold greater than the starting activity.

Table 2: Activity Of Porcine Cholesterol Esterase And Human BSSL: Lactoferrin Effect

| μg Lactoferrin Added | Activity, μmole pNPB/min/mg enzyme | |
|---|---|---|
| | Porcine CEase* | Human BSSL** |
| 0 | 81.0 | 6.2 |
| 4 | -- | 5.5 |
| 8 | 67.0 | 6.9 |
| 16 | 68.1 | -- |
| 20 | -- | 18.2 |
| 24 | 69.0 | -- |
| 32 | 63.4 | -- |
| 50 | -- | 13.1 |

*The assay contained $3.06 \times 10^{-4}$ mg porcine cholesterol esterase.
**The assay contained $1.0 \times 10^{-3}$ mg BSSL.

## EXAMPLE 4

This example shows that human lactoferrin enhances the BSSL catalyzed hydrolysis of either triolein or cholesteryl oleate when the lipid is packaged in a phosphatidylcholine vesicle (Cox *et al., Biochemistry, 29*:3842 (1990)). For these substrates, bile salt is an essential activator, and it must be included in the assay. Therefore, purified lactoferrin, increasing from 0 to 6 mg/mL, was added to the triolein assay mixture in the presence of 1.0 mM, 2.5 mM and 7.5 mM taurocholate (TC). The concentration of lactoferrin in the incubation mixture covered the range that is commonly found in human breast milk (Masson & Heremans, *Comp. Biochem. Physiol., 39B:*119-129 (1971); Hennart *et al., Am. J. Clin. Nutr., 53*:32 (1991)), while the bile salt concentration range was chosen to reflect those values found over the course of development. Thus, 7.5 mM, the value found in the older child, was chosen as a reference point, and its value was lowered to 2.5 mM and 1.0 mM in order to simulate the conditions commonly found in the infant (Watkins, *Pediatrics, 75(suppl.)*:151 (1985); Norman *et al., Acta Ped. Scand., 61*:571 (1972); Heubi *et al., J. Lab. Clin. Med., 100*:127 (1982)).

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The results in Table 3 below show the dramatic effect of bile salt on triolein hydrolytic activity (row 1). The BSSL catalyzed hydrolysis of triolein increased 25-fold from 1.6 μmole/hr/mg to 41.9 μmole/hr/mg when the bile salt increased from 1.0 mM to 7.5 mM in the reaction mixture. In addition, at 7.5 mM bile salt, increasing lactoferrin in the incubation mixture from 0 to 6 mg/mL, produced a modest 2.5-fold enhancement of activity from 41.9 μmole/hr/mg to 101.2 μmole/hr/mg (column 3). This effect is more striking at lower bile salt concentrations. Thus, in the presence of 2.5 mM taurocholate, 6 mg/mL lactoferrin stimulated hydrolytic acitivity ten-fold from 1.8 μmole/hr/mg to 18.5 μmole/hr/mg (column 2). Importantly, in the presence of high lactoferrin (6 mg/mL), the lipolytic activity in sub-optimal bile salt (2.5 mM) was only about two-fold less than the hydrolytic activity in the presence of optimal bile salt (7.5 mM) and no lactoferrin. These data demonstrate that lactoferrin can augment the activating effect of bile salt, allowing the enzyme to function at the low bile salt concentrations that are commonly found in the infant.

The data of Table 3 have been plotted in Figure 3 to show the BSSL activity versus the taurocholate concentration at various lactoferrin concentrations.

Table 3: Effect Of Lactoferrin On Triolein Hydrolysis: Bile Salt Dependence

| Lactoferrin, mg/mL | Activity (μmole oleate/hr/mg BSSL) | | |
|---|---|---|---|
| | 1.0 mM TC | 2.5 mM TC | 7.5 mM TC |
| 0 | 1.6 | 1.8 | 41.9 |
| 0.5 | 3.8 | 8.5 | 61.1 |
| 1 | 4.6 | 15.3 | 77.9 |
| 3 | 5.6 | 18.9 | 90.5 |
| 6 | 5.1 | 18.5 | 101.2 |

Since the BSSL catalyzed hydrolysis of cholesteryl oleate is also bile salt dependent, the effect of lactoferrin on this reaction was also examined. As shown in the table below, similar results were found. In this case, hydrolytic activity in the presence of low bile salt (2.5 mM) and high lactoferrin (6 mg/mL), 25.7 μmole/hr/mg, was actually greater than that found in high bile salt (7.5 mM) and no lactoferrin, 19.8 μmole/hr/mg. Taken together, both these assay systems demonstrate that lactoferrin interacts with bile salt/vesicles and/or enzyme to enhance lipase activity in the presence of sub-optimal concentrations of bile salt.

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Table 4: Effect Of Lactoferrin On Cholesteryl Oleate Hydrolysis: Bile Salt Dependence

| Lactoferrin, mg/mL | Hydrolytic Activity* | |
|---|---|---|
| | 2.5 mM Taurocholate | 7.5 mM Taurocholate |
| 0 | 9.8 | 19.8 |
| 0.5 | 13.5 | 24.4 |
| 1 | 15.3 | 25.1 |
| 3 | 21.5 | 32.7 |
| 6 | 25.7 | 34.1 |

*μmole oleate/hr/mg BSSL

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinency of the cited references.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

What is claimed is:

1. A dietary composition having enhanced digestibility of long chain triglycerides by infants and other subjects with a deficiency of bile salt, the composition comprising long chain triglycerides and lactoferrin.

2. A dietary composition as set forth in claim 1, wherein the long chain triglycerides are from one source and the lactoferrin is from a second source.

3. A dietary composition as set forth in claim 1, wherein the lactoferrin is human lactoferrin.

4. A dietary composition as set forth in claim 3, wherein the human lactoferrin is isolated human lactoferrin.

5. A dietary composition as set forth in claim 4, wherein the human lactoferrin is isolated from human milk.

6. A dietary composition as set forth in claim 5, wherein the human lactoferrin is produced by a recombinant organism.

7. A dietary composition as set forth in claim 6, wherein the recombinant organism is a recombinant microorganism.

8. A dietary composition as set forth in claim 7, wherein the recombinant organism is a transgenic animal.

9. A dietary composition as set forth in claim 3, wherein the triglycerides are derived from milk.

10. A dietary composition as set forth in claim 9, wherein the triglycerides are derived from bovine milk.

11. A dietary composition as set forth in claim 3, wherein the triglycerides are non-milkfat triglycerides.

12. A dietary composition as set forth in claim 1, wherein the composition further comprises BSSL.

13. A dietary composition as set forth in claim 12, wherein the BSSL is isolated BSSL.

14. A dietary composition as set forth in claim 13, wherein the bile salt stiumlated lipase is human BSSL.

15. A dietary composition as set forth in claim 3, wherein the lactoferrin is present in an amount sufficient to at least double the rate of hydrolysis of the triglycerides when such composition is ingested by a human infant, where doubling

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the rate refers to a rate at least twice that obtained for the same composition, but free of lactoferrin.

16. A dietary composition as set forth in claim 15, wherein the lactoferrin is present in an amount that provides a lactoferrin concentration of at least about 1 mg/ml in the small intestine of the human infant.

17. A dietary composition as set forth in claim 15, wherein the lactoferrin is present in an amount that maximizes the rate of hydrolysis of the triglycerides for the limited amount of bile salt that is present.

18. Human infant formula having enhanced fat digestibility, comprising bovine milk triglycerides and isolated human lactoferrin.

19. Human infant formula having enhanced fat digestibility, comprising soy milk triglycerides and isolated human lactoferrin.

20. Human infant formula as set forth in claim 18, further comprising isolated human BSSL.

21. Fortified milk composition comprising long chain triglycerides from a first source and isolated lactoferrin from a second source in an amount sufficient to result in a rate of hydrolysis of the triglycerides when the fortified milk composition is contacted with BSSL and bile salt that is double what results if a lactoferrin-free composition that is otherwise identical to the fortified milk composition is contacted with BSSL and bile salt.

22. A method for enhancing the digestion and absorption of long chain triglycerides, the method comprising fortifying the triglycerides with an amount of lactoferrin sufficient to double the rate of hydrolysis of the triglycerides over the rate obtained in the absence of lactoferrin, when such triglycerides and lactoferrin are in the presence of BSSL and in the presence of a deficiency of bile salt.

23. A method for enhancing the BSSL hydrolytic activity of triglycerides in the presence of a deficiency of bile salt comprising contacting the BSSL with long chain triglycerides in the presence of the bile salt and an amount of lactoferrin sufficient to double the rate of triglyceride hydrolysis, over the rate of hydrolysis obtained in the absence of lactoferrin, and wherein the bile salt is present in a concentration of less than its CMC.

24. A method as set forth in claim 23, wherein the bile sale is present in a concentration of less than about 3 mM.

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25. A method for treating a subject having a bile salt deficiency, the method comprising administering to the subject, in conjunction with the oral ingestion of long chain triglycerides, bile salt stimulated lipase and isolated lactoferrin in an amount sufficient to double the rate of digestion of the triglycerides over the rate obtained in the absence of lactoferrin and to increase absorption of the fats by the subject.

26. A method for feeding an infant a dietary base from a first source, which base contains long chain triglycerides, the method comprising administering BSSL and human lactoferrin to the infant in an amount sufficient to improve the infant's digestion and absorption of the triglycerides, wherein the lactoferrin is from a second source.

27. A method of preparing a dietary composition having enhanced digestibility of long chain triglycerides comprising mixing with such triglycerides an amount of lactoferrin sufficient to improve the digestion and absorption of the triglycerides.

28. A method as set forth in claim 27, further comprising mixing bile salt stimulated lipase together with said composition.

29. A method as set forth in claim 27, further comprising mixing with said composition isolated bile salt stimulated lipase.

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# S-SEPHAROSE CHROMATOGRAPHY OF BSSL

(2.6x5.5 cm; 63 mL/hr; 150 mL Gradient;
300 mL NaCl, 25 mM Ac pH 5.1 to 1.0 M NaCl, 25 mM Ac pH 5.1)

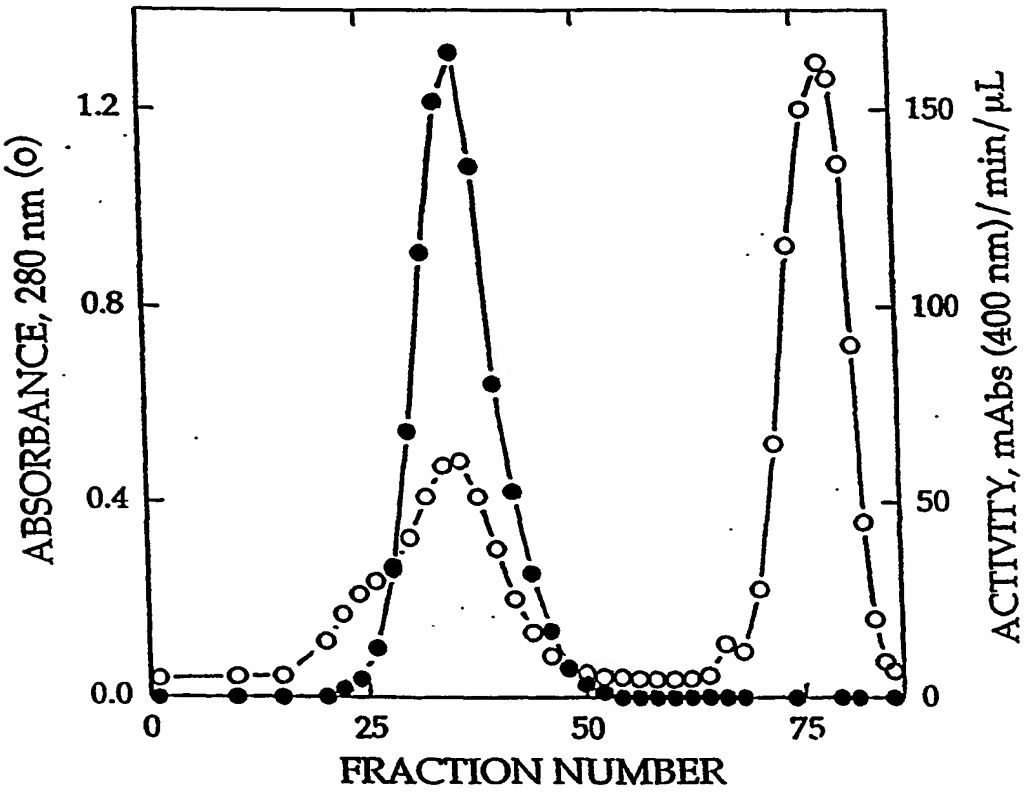


**FIGURE 1.**

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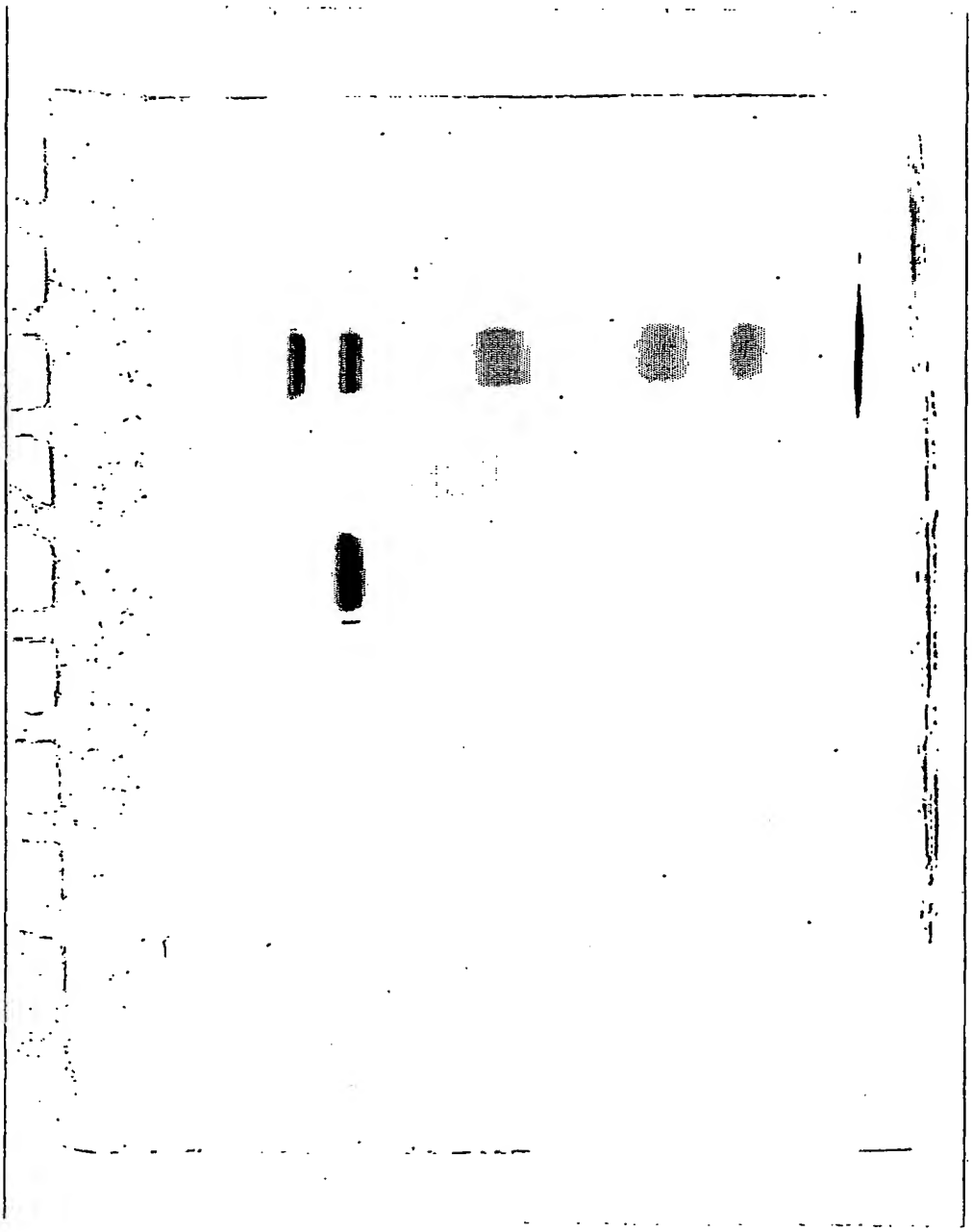

FIGURE 2

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EFFECT OF LACTOFERRIN ON THE HYDROLYSIS OF TRIOLEIN BY BILE SALT STIMULATED LIPASE

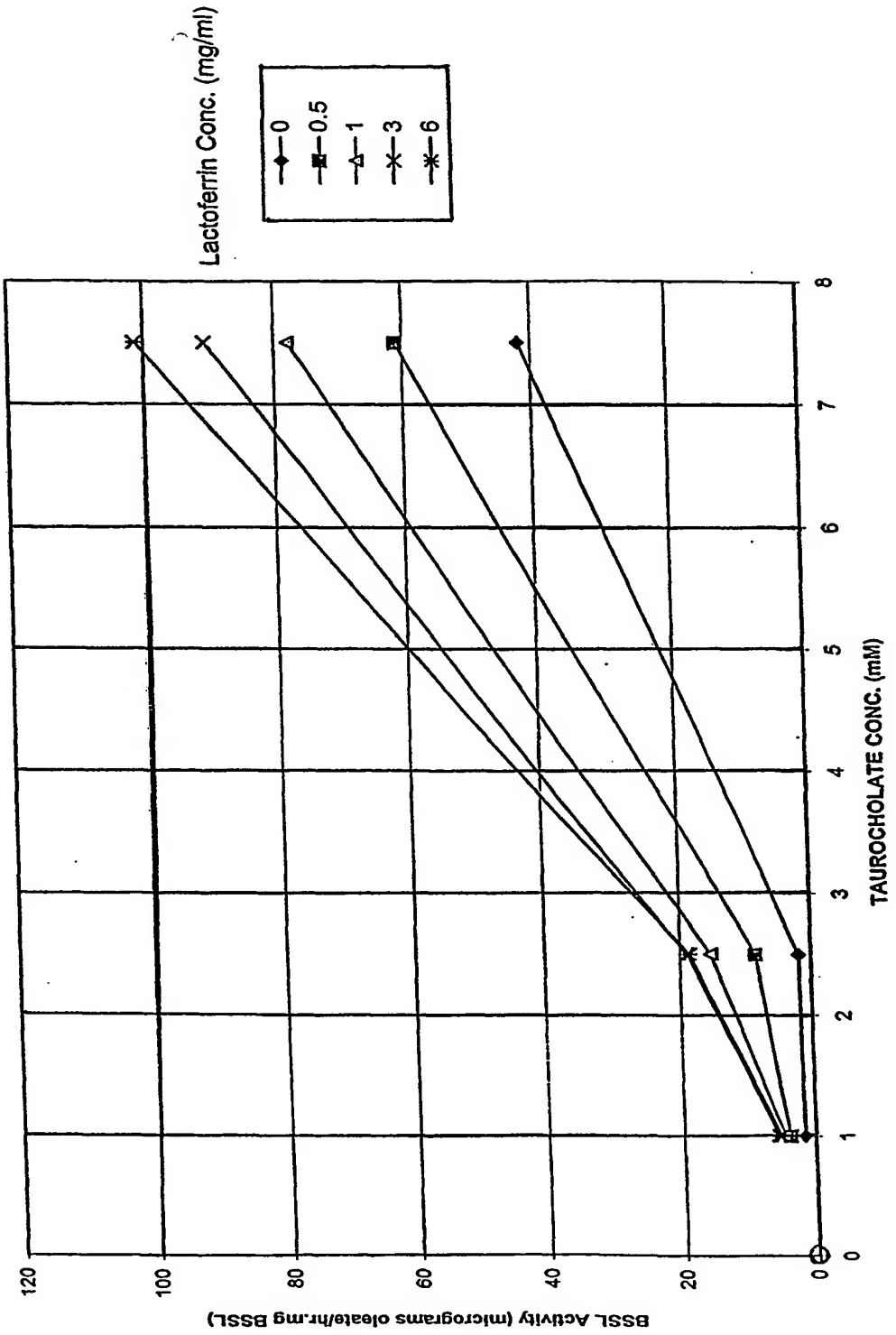


FIGURE 3.

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/13836

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :A61K 38/14; 38/02; A23L 1/305
US CL :514/21; 426/34, 35, 42, 580
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/21; 426/34, 35, 42, 580

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, DIALOG
search terms: bile salt, milk, infant, baby, formula, dairy, lactoferrin, bile salt stimulate (activated) lipase, BSSL

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 4,977,137 A (NICHOLS et al.) 11 December 1990, see entire document. | 1-29 |
| Y | US 4,944,944 A (TANG et al.) 31 July 1990, see entire document. | 1-29 |
| Y | US 5,716,817 A (TORNELL) 10 February 1998, see entire document. | 1-29 |

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
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| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 03 JULY 2000 | 11 AUG 2000 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>KEITH D. HENDRICKS<br>Telephone No. (703) 308-0661 |

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